Title: Cloning LuxI-RFP on pSB1C3

Goal: To get LuxI and RFP on the same backbone
Date range: June - September, 2015
Members that worked on project: Naomi Iverson

Results:
LuxI-RFP on a contaminated 1C3 backbone due to initial contamination from BBa_C0061 part.

New part number: This part was not submitted

Lengths of parts:
BBa_C0061 (LuxI portion of plasmid) = 643bp
RBS-RFP = 12bp + 706bp = 718bp
pSBIC3 = 2070bp

Transformation:

- Pulled BBa_C0061 (LuxI on 1C3) and BBa_J04450 (RFP on 1C3) from iGEM kit.
- 1 ul of plasmid from iGEM kit into 40ul of 5 alpha competent cells.
- Let cells sit with plasmid on ice for 30 minutes.
- Heat shock at 42°C for 45 seconds.
- Put on ice for 5 minutes.
- Treat cells with 950ul of SOC.
- Put in incubator at 37°C for 1 to 2 hours.
- Spread 100ul of cells onto chloramphenicol resistance plates (170ng/ul concentration)

Overnight:

- Make overnights with 5mL of LB and 25ul chloramphenicol.

Mini-prep:

- BBa_C0061 concentration: 417.8ng/ul.
- BBa_J04450 concentration: 249ng/ul.

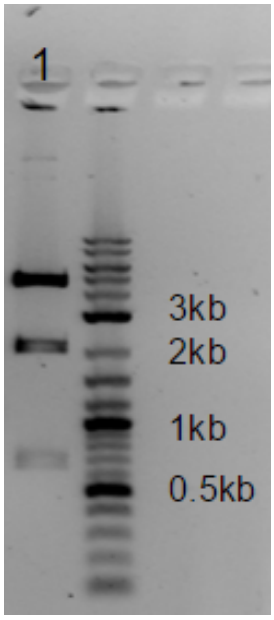
Gel of BBa_C0061 after mini-prep:

- Ladder: 2-log
- Gel concentration: 1%

BBa_C0061 Digest
(June 26st, 2015)

Lane 1: C0061 cut with EcoRI and PstI. Note: the largest band is some form of contamination as it does not match any expected lengths from BBa_C0061.

C0061 total ~2713bp
1C3 ~2070bp
LuxI ~643bp



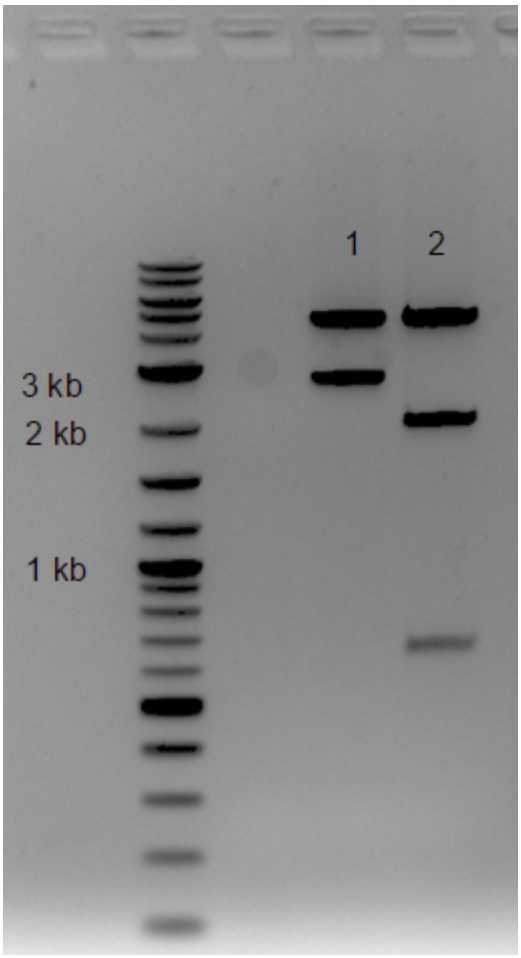
Thinking that we had contaminated BBa_C0061, in late July we order a new kit with fresh BBa_C0061. However, the fresh BBa_C0061 product also produced the exact same contamination bands as the initial BBa_C0061:

BBa_C0061 Digest
(July 21st, 2015)

Lane 1: C0061 cut with EcoRI. Note: the largest band is some form of contamination as it does not match any expected lengths from BBa_C0061.

Lane 2: C0061 cut with EcoRI and PstI. Note: the largest band is some form of contamination as it does not match any expected lengths from BBa_C0061.

C0061 total ~2713bp
1C3 ~2070bp
LuxI ~643bp



This contamination created many difficulties during the cloning for us and ultimately prevented us from making a clean LuxI-RFP-1C3 plasmid.

PCR of BBa_J04450:

- Purpose: Get the RFP gene off from J04450 and to add the RBS site in front of the gene.
- PCR polymerase: Q5 HF 2x Master Mix.
- Primers used: GEM027 Forward and Reverse.

Reagent	25 ul reaction	Concentration
2x Q5	12.25 ul	1 x
10 uM F. Primer	1.25 ul	0.5 uM
10 uM R. Primer	1.25 ul	0.5 uM
DNA (249.0 ng/ul)	1 ul	0.249 ng
Q5 polymerase	0.25 ul	0.02 U/ul
Water	9.0 ul	

Reaction conditions:

Step	Temp (C)	Time
I. Denature	98	30 s.
35 cycles	98	10 s
	64	15 s
	72	20 s
Final extension	72	2 min.

- Annealing temp: 64°C
- Extension time: 20 s

PCR clean-up:

- Elution volume: 30ul
- Concentration: 66.0 ng/ul

PCR of BBa_C0061:

- Purpose: Add the RBS site in front of LuxI gene.
- PCR polymerase: Q5 HF 2x Master Mix.
- Primers used: GEM033 Forward and GEM025 Reverse.

Reagent	25 ul reaction	Concentration
2x Q5	12.25 ul	1 x
10 uM F. Primer	1.25 ul	0.5 uM
10 uM R. Primer	1.25 ul	0.5 uM
DNA (417.8 ng/ul)	1 ul	0.418 ng
Q5 polymerase	0.25 ul	0.02 U/ul
Water	9.0 ul	

Reaction conditions:

Step	Temp (C)	Time
I. Denature	98	30 s.
35 cycles	98	15 s
	64	20 s
	72	30 s
Final extension	72	2 min.

- Annealing temp: 64°C
- Extension time: 30 s

Digestion of RBS-LuxI PCR product:

- Purpose: digest at E and P cut sites to make sticky ends for ligation with 1C3 backbone
- Enzymes used: EcoRI-HF and PstI-HF

Reagent	50 ul reaction	Concentration
Cutsmart Buffer	5 ul	1 x
EcoRI-HF	1 ul	10 units
PstI-HF	1 ul	10 units
DPN1 (since PCR product)	1 ul	10 units
DNA (121.2 ng/ul)	4 ul	500 ng
Water	39 ul	

Digestion of 1C3:

- Purpose: digest at E and P cut sites to make sticky ends for ligation with RBS-LuxI
- Enzymes used: EcoRI-HF and PstI-HF

Reagent	50 ul reaction	Concentration
Cutsmart Buffer	5 ul	1 x
EcoRI-HF	1 ul	10 units
PstI-HF	1 ul	10 units
DNA (66.0 ng/ul)	7.58 ul	500 ng
Water	35.42 ul	

Ligation:
Vector = 1C3
Insert = RBS-LuxI

Component	20 ul reaction	Concentration/amount
10XT4 DNA Ligase Buffer	2 ul	1 x
Vector DNA (2070 bp)	26.9 ng	0.02 pmol
Insert DNA (~650 bp)	25 ng	0.06 pmol
T4 DNA Ligase	1 ul	
Nuclease-free water	To 20 ul	

Let reaction run for 12 hours at 16°C then heat shock at 65°C for 10 minutes.

Then perform transformation by using previously described transformation procedure. Only difference: instead of using 1 ul of DNA, put 10 ul of DNA into a tube of 40 ul of alpha 5 competent cells.

Grew up 3 overnights after letting cells grow on plates overnight.

Mini prepped all 3 overnights of RBS-LuxI-1C3, digested with E and P (followed same digestion procedure as noted above) and ran on gel:

LuxI-1C3
(July 7, 2015)

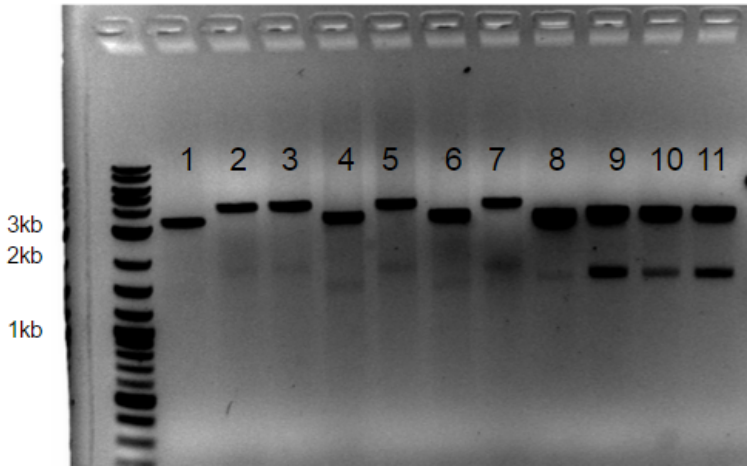
Lane 9: LuxI-1C3 digested with EcoRI, colony 1

Lane 10: LuxI-1C3 digested with EcoRI, colony 2

Lane 11: LuxI-1C3 digested with EcoRI, colony 3

Note the contamination once again (two bands instead of one expected band at roughly 2713bp).

1C3 ~2070bp
LuxI ~643bp



Digested LuxI-1C3 colonies 2 and 3 following the same digestion steps as noted above. Used SpeI and PstI enzymes.

Digested RBS-RFP following the same digestions steps as noted above. Used XbaI and PstI enzymes.

Ligation of LuxI-1C3 to RBS-RFP

Transformed ligation product using same protocol as mentioned above.

Colonies grew on plate, however most colonies fluoresced red. This would infer that an RFP promoter exists within the plasmid, however no such promoter should exist on this plasmid since we PCRed the RFP straight out of BBa_J04450 and put it directly into the LuxI-1C3 plasmid. Therefore, something else (a contaminating factor) inserted itself into the plasmid that may have caused RFP to be transcribed. This contaminating factor most likely came from the original contamination in BBa_C0061.

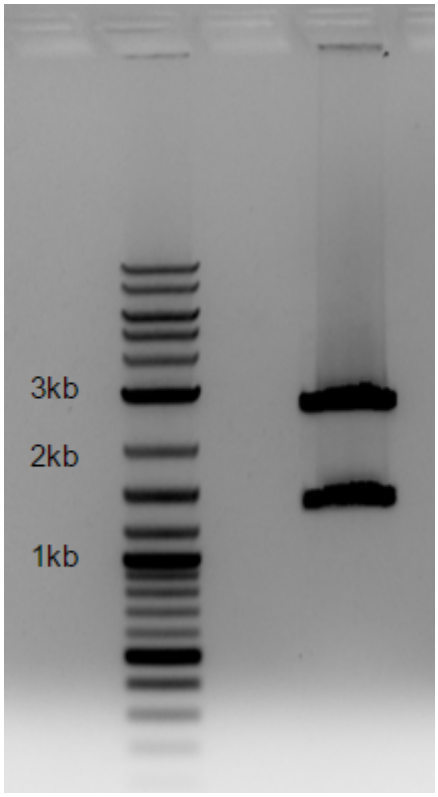
Made overnights and mini-prepped the next day.

Digested LuxI-RFP-1C3 mini-preps with E and P and ran on gel:

LuxI-RFP-1C3
(August 19th, 2015)

It appears that while the insert looks very close to the correct length (~1361bp), the vector (1C3 backbone) appears to be longer than 2070bp. Instead, it is nearly at the 3kb mark. This indicates that something has integrated itself into the 1C3 backbone and may have caused the transcription of RFP.

1C3 ~ 2070bp
LuxI ~ 643bp
RFP ~ 718bp
LuxI+RFP ~ 1361bp



We have attempted to gel extract the insert and ligate it onto a clean 1C3 backbone, however we have had zero success with this to date for unknown reasons.

Therefore, we have not succeeded (with the time allotted this summer) to construct our LuxI-RFP-1C3 construct due most likely to contamination existing originally within BBa_C0061.